ORIGINAL ARTICLE

Monitoring the genotype of meningococcal strains during an endemic period

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Objective: To monitor the circulation of clones among the most frequent phenotypes of *Neisseria meningitidis* isolated from sporadic cases in Italy in recent years.

Methods: *N. meningitidis* strains sent to the reference laboratory for meningococcal meningitis of the Istituto Superiore di Sanità were typed for serogroup, serotype and serosubtype, and chromosomal DNAs were analyzed by pulsed-field gel electrophoresis.

Results: Of 185 strains sent during the years 1995–97, 75% belonged to serogroup B. Although many different serotypes and subtypes were present, the most common combinations were B:14:P1.13, B:4:P1.13, B:15:P1.7 and C:2b:P1.2. PFGE analysis of patterns obtained by using *Nhe*I and *Bgl*II enzymes showed a quite stable genomic structure, except in the phenotype B:4:P1.13.

Conclusions: On the basis of serotype and serosubtype analysis of recently isolated meningococci, a great variety of phenotypes has been circulating during these endemic years. A clonal structure in strains belonging to the same phenotypes was found, suggesting that some favorable conditions have led to stability of these highly transmissible strains.

Key words: Meningococci, serotypes, PFGE

INTRODUCTION

Over the last 15 years, the trend of meningococcal disease in Italy has been that of a low endemic period, with a mean attack rate of 0.4/100 000 per year [1–3]. Between 1984 and 1989, a predominance of *Neisseria meningitidis* C:2a:P1.2 was observed and, by multilocus enzyme electrophoresis, about 70% of the isolates were assigned to one of four very closely related electrophoretic types named ET37 complex [4]. Since 1989, a change in the epidemiologic pattern of meningococcal isolates has occurred, with a dramatic shift from serogroup C to serogroup B [3]. The most common antigenic phenotypes now circulating in Italy are B:4:P1.13, B:14:P1.13, and B:15:P1.7.

Recent studies [5,6] suggest that meningococcal populations, other than serogroup A isolates, are basically non-clonal, but that hyperendemic strains arise at intervals and predominate. In recent years, the genotypic data obtained by the use of pulsed-field gel electrophoresis (PFGE) have been shown to be particularly reliable in establishing relatedness or identity among meningococcal isolates [7,8]. Within the National Program for surveillance of meningococcal meningitis, PFGE was used to monitor the circulation of clonal types among the most frequent meningococcal phenotypes collected over a 3-year period.

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MATERIALS AND METHODS

Bacterial strains

The Italian Reference Laboratory (RL) for bacterial meningitis of the Istituto Superiore di Sanità collects *N. meningitidis* isolates from cases of meningitis from all over the country. In the period 1995–97 the RL received 185 isolates (72 in 1995, 51 in 1996, and 62 in 1997) for typing.

Serotyping and serosubtyping

Serogroup B and serogroup C meningococci were further typed according to the class 2 and 3 outer-membrane proteins (OMPs) and to the class 1 OMPs to define the serotype and the serosubtype respectively.

A whole cell enzyme-linked immunosorbent assay was used [9], and the panel of monoclonal antibodies for serotyping and serosubtyping was provided by the National Institute for Biological Standards and Control (UK).

DNA preparation for PFGE analysis

DNA was prepared according to the method described by Matushek et al [10], with some minor modifications. Bacteria were grown on Thayer–Martin medium for 24 h at 37°C in a candle jar. The cells were removed from the plates and suspended in 2–3 mL of sterile TE (Tris-HCl 10 mM, EDTA 1 mM, pH 8.0) to produce a turbidity equivalent to McFarland 2. The organisms were inactivated at 56°C for 1 h and stored at 4°C for 24 h. An aliquot of this suspension (500 μL) was mixed immediately with an equal volume of tempered 1.6% low-melting-point-agarose, LMP (Sigma Chemical Co., St Louis, Mo, USA), vortexed briefly, pipetted into plug molds (capacity 100 μL) (Bio-Rad Laboratories, Hercules, Ca, USA) and allowed to solidify. After lysis, the plugs were incubated with 15 mL of ESP solution (0.5 M EDTA, pH 8, 1% sarkosyl and 1 mg/mL proteinase K), and incubated overnight at 50°C. After washing with TE buffer, the digestion by restriction endonucleases was performed in a total volume of 200 μL, with 30 U of two different endonucleases, *Nhe*I and *Bgl*II (New England Biolabs, Beverly, Ma, USA), overnight at 37°C.

Pulsed-field gel electrophoresis

Digested plugs were sealed into slots in a 1% agarose gel (Bio-Rad Laboratories) in a CHEF-MAPPER II (Bio-Rad Laboratories) apparatus in 0.5 TBE (45 mM Tris-HCl, 45 mM boric acid, 1 mM EDTA) buffer at 14°C. A voltage of 4.5 V/cm was used, with a pulse time of 1 s to 30 s for 24 h. The gels were stained with 0.5 mg/L of ethidium bromide, viewed under ultraviolet light, and photographed.

Analysis of data

An assessment of the degree of similarity between fingerprinting patterns was obtained by comparing patterns and scoring the number of shared bands. Following the criteria indicated by Dice [11], isolates were considered to be related if the coefficient of similarity (CS) was 0.85–0.99, and different if the CS was <0.85. The guidelines and interpretive criteria described by Tenover et al [12] were also used to analyze restriction patterns and to identify relatedness among isolates.

RESULTS

The 185 isolates sent to the RL during the years 1995–97 represent approximately one-third of meningococcal strains responsible for meningitis during that period and 75% of all the meningococcal strains isolated in hospital laboratories according to the data from the National Surveillance of bacterial meningitis.

The trend of meningococcal disease is that of a low endemic period, and the predominance of serogroup B meningococci still persists [3] after a shift from C to B which took place at the beginning of the 1990s: the average proportion of meningococci belonging to serogroup B (75%) is high compared to serogroup C (20%). Even in the presence of a large distribution of different phenotypes in the country, the most common combinations of serotypes and serosubtypes in these two predominant serogroups are B:4:P1.13 (6.5%), B:14:P1.13 (7.0%), B:15:P1.7 (3.8%), C:2a:P1.2 (3.7%) and C:2b:P1.2 (4.3%). The percentages of these last two phenotypes also include strains where the P1.2 subtype is associated with subtypes P1.5 and P1.6. *N. meningitidis* B:14:P1.13 seems to be circulating predominantly in the northeastern regions, while B:4:P.13 and B:15:P1.7 have a more uniform distribution. Similarly, C:2a:P1.2 is present in the north and has never been detected in the southern regions, whereas C:2b:P1.2 is more frequent in the south. Serotyping based on OMPs of class 2/3 and class 1 produced 3.8% and 4.9% of non-typable strains, respectively.

All the 47 strains belonging to the five more common phenotypes of disease-associated *N. meningitidis* were examined by PFGE using the *Nhe*I and *Bgl*II restriction endonucleases. Table 1 shows the pulse types (PTs) recovered by using *Nhe*I, the more discriminatory of the two enzymes. In particular, the B:14:P1.13 strains, isolated mostly in the northeastern area of the country, show an identical genomic pattern, PTA, even if they were isolated from sporadic cases that occurred between 1995 and 1997. Figure 1A shows representative examples of this PTA. The strains also showed a CS >0.85 when analyzed after digestion with *Bgl*II (data not shown), suggesting the spread of a single clone within this phenotype.

All six strains belonging to the B:15:P1.7 phenotype showed profiles with a limited diversity consistent with one independent genetic event, and the isolates were considered to be the closely related PTB and PTB1 (Figure 1B, lanes 1 and 2), while only one strain, with a subtype P1.1,7 (PTC), showed a completely different profile (Figure 1B, lane 3).

The *Nhe*I fingerprints of the 12 B:4:P1.13 strains isolated in different geographic areas of the country show the presence of different genomic patterns (PTD–PTG). Up to five or six different fragments were identified, indicating changes produced by three genetic events in two-thirds of the strains. Thus, the majority of the isolates showing this phenotype were considered

Table 1 Pulse types of the five most common phenotype combinations detected in *N. meningitidis* strains isolated in Italy from 1995 to 1997

Phenotype	No. of strains	Pulse type
B:14:P1.13	13	A
B:15:P1.7	4	B
	2	B1
B:15:P1.1,7	1	C
B:4:P1.13	4	D
	3	E
	1	F
	4	G
C:2a:P1.2	3	H
C:2a:P1.2,5	3	H1
C:2a:P1.5	1	I
C:2b:P1.2	5	L
C:2b:P1.2,5	2	L1
C:2b:P1.2,6	1	L2

to be unrelated or, at the most, possibly related. Figure 1B (lanes 4–7) shows the representative examples of DNA fingerprints identified in this phenotype.

Finally, fingerprinting profiles of strains belonging to serogroup C showed clonality among those with serotype 2a or serotype 2b, subtype P1.2. The six strains C:2a:P1.2 or C:2a:P1.2,5 examined with *Nhe*I (Figure 2, lanes 1 and 2) and *Bgl*II (data not shown) shared closely related patterns, as indicated in Table 1 (PTH, PTH1). The different pattern (PTI) produced by an unrelated phenotype, C:2a:P1.5, is shown in Figure 2, lane 3. Interestingly, the pattern showed by recently isolated C:2a:P1.2 strains appears to be identical to the fingerprinting of C:2a:P1.2 strains isolated 10 years ago (data not shown), which, by a different technique, had at the time already been shown to be a single clone [4].

The five strains with the phenotype C:2b:P1.2 showed identical patterns (PTL), represented in Figure 2, lane 4, closely related to those (PTL1, PTL2) produced by phenotypes C:2b:P1.2,5 or P1.2,6 (Figure

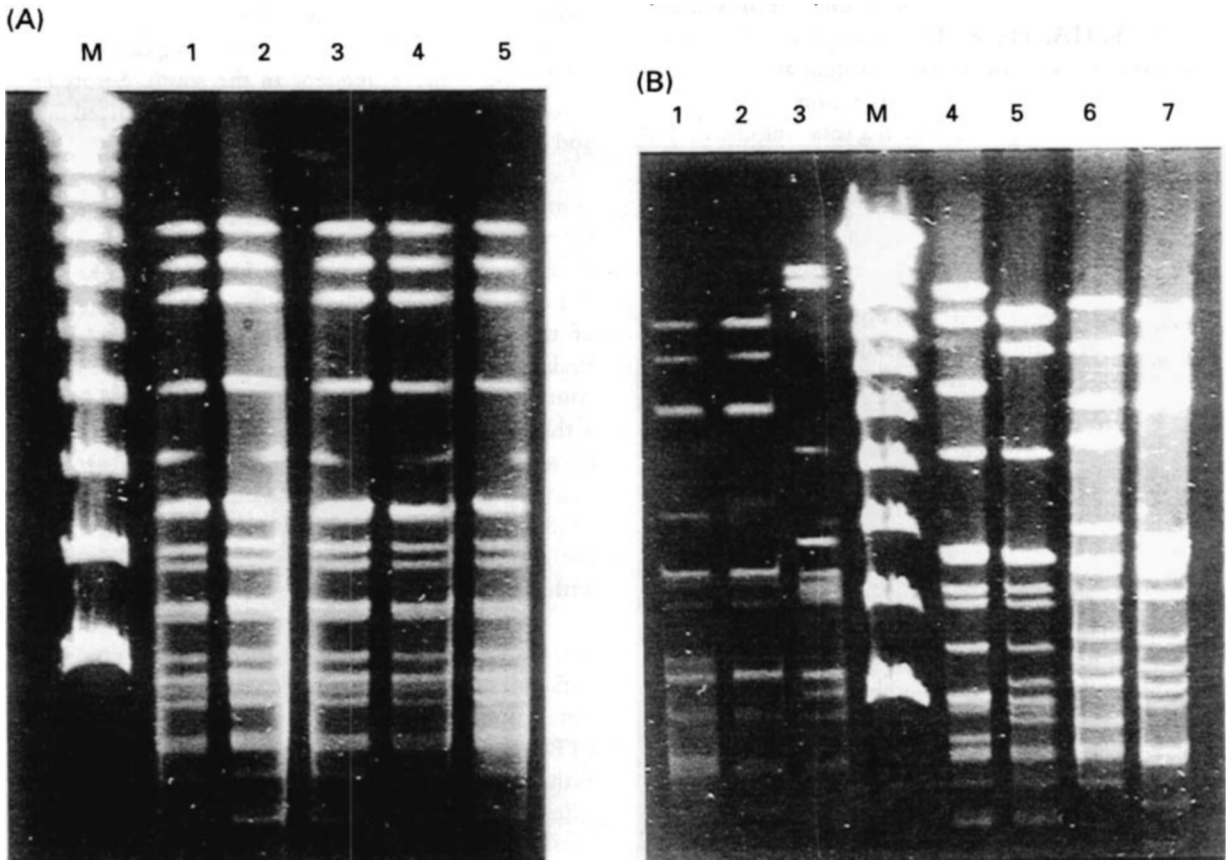

Figure 1 Genomic fingerprinting of *Neisseria meningitidis* serogroup B clinical isolates by PFGE using *Nhe*I restriction enzyme. (A) Lanes 1–5: examples of the identical profiles (PTA) produced by cleaved genomic DNAs of *N. meningitidis* B:14:P1.13. Lane M: lambda ladder molecular size marker (New England Biolabs). (B) Lanes 1 and 2: examples of the clonal pulse types, PTB and PTB1, produced by the cleaved genomic DNAs of *N. meningitidis* B:15:P1.7. Lane 3: B:15:P1.7 strain showing completely different profile, PTC. Lanes 4–7: examples of the different pulse types PTD, PTE, PTF and PTG produced by *N. meningitidis* B:4:P1.13. Lane M: lambda ladder molecular size marker (New England Biolabs).

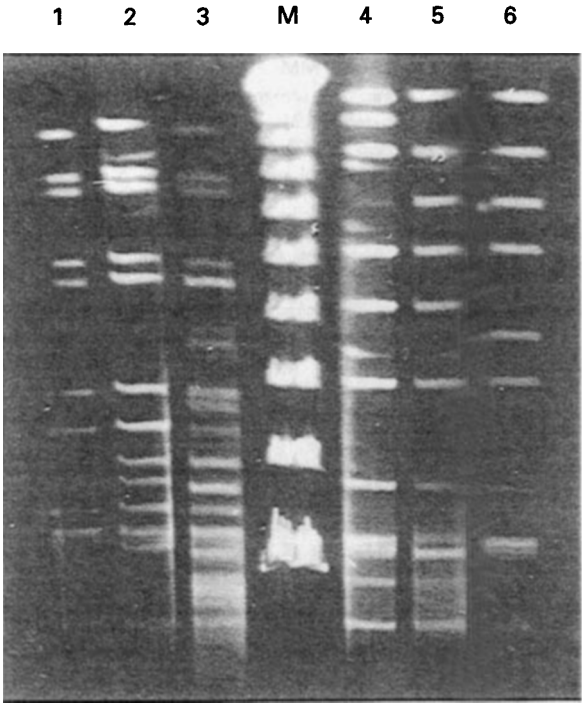

Figure 2 Genomic fingerprinting of *N. meningitidis* serogroup C clinical isolates by PFGE using *Nhe*I restriction enzyme. Lanes 1 and 2: examples of patterns produced by *N. meningitidis* C:2a:P1.2 (PTH) and C:2a:P1.2,5 (PTH1), respectively. Lane 3: different pattern (PTI) produced by the unrelated phenotype C:2a:P1.5. Lanes 4–6: closely related patterns PTL, PTL1 and PTL2 of *N. meningitidis* C:2b:P1.2, C:2b:P1.2,5 and C:2b:P1.2,6 respectively. Lane M: lambda ladder molecular size marker (New England Biolabs).

with the pattern PTL, were resistant to rifampin, with a minimum inhibitory concentration (MIC) >256 mg/L by the Etest (AB Biodisk, Solna, Sweden). The only other rifampin-resistant strain of the 185 (MIC >256 mg/L), typed as C:2b:P1.10, showed a different profile (not shown).

DISCUSSION

Meningococcal meningitis and septicemia occur endemically in most of western Europe and North America, affecting mainly infants, young children and teenagers [13,14]. In Italy, the incidence of the disease is low compared with other European countries, where, between 1993 and 1996, an increased incidence was seen [15]. During the 1980s, a low endemic period in our country, we experienced the spread of a single clone which became prevalent: about 70% of meningococcal isolates were assigned to one of four closely related electrophoretic types, named ET37 complex [4]. While the low endemic period still persists, since the beginning of this decade a new epidemiologic pattern has appeared, with a shift in the predominant serogroup, from C to B [3]. This increase in serogroup B relative to C has also been observed in most other European countries [15]. On the basis of serotype and serosubtype analysis of strains collected within the National Surveillance Program, a great many phenotypes are now circulating in the country, and the five most frequent combinations (B:4:P1.13, B:14:P1.13, B:15:P1.7, C:2a:P1.2, C:2b:P1.2) represent only 21.6% of all meningococcal isolates. Since, however, *N. meningitidis* serogroups B and C are highly transformable organisms, the persistence over time and distance of similar antigenic phenotypes might suggest the presence of epidemic characteristics in these strains. To discriminate among these *N. meningitidis* strains, we analyzed their genetic relationships by PFGE, using mainly the rare cutting enzymes *Nhe*I and *Bgl*II, since this method is widely accepted as having good resolution [7,16]. The analysis of pulse types within each of the five more common phenotype combinations showed a quite stable genomic structure in at least four of them. In particular, the strains belonging to phenotype B:14:P1.13 showed a single predominant PFGE type over the 3-year time period and a specific concentration (83%) in the northeastern area of the country. Almost the same picture was provided by the B:15:P1.7 strains, which shared similar patterns although the isolates came from different areas. Conversely, the more widely disseminated B:4:P1.13 strains show a low degree of genetic relatedness, with the circulation of possibly related or unrelated strains. *N. meningitidis* strains belonging to the phenotype C:2a:P1.2, which predominated in Italy as a clonal population during the 1980s [4], are now less frequent, having been replaced by other subtypes and serotype 2b. Interestingly, the analysis of the pulse types shows that strains isolated recently still present the same genomic pattern as the strains isolated 10 years ago.

Overall, these data seem to indicate limited rearrangements in the chromosome of strains belonging to the same phenotype (serogroup, serotype, subtype), suggesting that some favorable conditions lead to the stability of highly transmissible strains, although the period of observation in this study, 3 years for group B meningococci and 10 years for group C, may be considered short in terms of population genetics. Knowledge of the circulation of phenotypes with a clonal structure in the meningococcal population of a country, even during endemic periods, may be useful as part of a routine surveillance of disease-associated isolates. In fact, the combination of serologic typing with a relatively simple genomic typing technique such as PFGE, as shown also in other studies [17,18], allowed

us to monitor both old and new features in the meningococcal population.

Although multilocus enzyme electrophoresis is the most widely used technique to study the epidemiology of meningococci and to estimate levels of genetic diversity within populations in the world [5], PFGE has the advantage of identifying subclones within the same electrophoretic type [19]. One of the drawbacks of PFGE, however, is that results obtained in different laboratories cannot be compared easily. In the future, the use of new approaches in molecular typing, such as the very recently described multilocus sequence typing [20], will overcome this limitation, and provide a global picture of the population structure of meningococci.

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